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PRINCIPAL INVESTIGATOR: Eileen Rogan, Ph.D.

CONTRACTING ORGANIZATION: Garvan Institute of Medical Research
Darlinghurst NSW 2010 Australia

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Eileen Rogan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Garvan Institute of Medical Research
Darlinghurst NSW 2010 Australia

E-MAIL:
e.rogan@garvan.unsw.edu.au

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Estrogenic steroids have diverse physiological functions and are critically involved in the pathogenesis of breast cancer. The transcriptional regulator c-Myc has been implicated in estrogen-induced mitogenesis and we are therefore investigating the contribution of c-Myc to various downstream molecular and cellular events after estrogen stimulation. Transcriptional activation of c-*myc* is an early response to estrogen treatment and we have previously shown that induction of c-Myc quantitatively reproduces the effects of estrogen on cell cycle progression. Furthermore, c-Myc induction mimics other downstream effects of estrogen including activation of cyclin E-Cdk2 by decreased association with the CDK inhibitor p21. Accumulating evidence suggests that the transforming capacity of c-Myc correlates more closely with transcriptional repression than with activation, raising the question of which function is the more important for c-Myc regulation of cell cycle progression. Our ongoing experimentation is thus aimed at using c-Myc mutants lacking specific functional domains to identify the domains that are important for estrogen-induced G_1 phase progression and the c-Myc target genes that mediate their effects. A panel of suitable constructs has now been generated and validated. These will be transfected into MCF-7 cells to assess their ability to recapitulate estrogen effects on parameters including cell cycle progression and cyclin E-Cdk2 activation. These experiments are expected to facilitate identification of the subset of c-Myc targets which are essential for estrogen-regulated proliferation.

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Table of Contents

INTRODUCTION

This project focuses on the role of c-Myc in estrogen induction of proliferation in breast epithelial cells. Specifically, the aim is to determine the contribution of c-Myc to various downstream molecular and cellular events after estrogen stimulation. The rationale for this investigation lies in: the established intrinsic role of estrogens in mammary gland development and etiology of breast cancer; the proven therapeutic efficacy of antiestrogens in breast cancer; and compelling evidence that has implicated the cell cycle regulatory molecules c-Myc and cyclin D1 in estrogen-induced mitogenesis. Our experimental approach involves ectopic expression of wild-type, mutant and dominant-negative variants of c-Myc to modulate c-Myc function and assay the necessity for various functional domains of c-Myc in mediating estrogen stimulation of breast cancer cell proliferation. An excellent model previously established in this laboratory has facilitated observation of various molecular events downstream from estrogen in breast cancer cells: antiestrogen-arrested cells synchronously re-enter the cell cycle after treatment with estrogen. Induction of c-Myc can also stimulate re-entry into the cell cycle and induction of various cell cycle regulatory proteins, formation and activation of regulatory protein kinase complexes, phosphorylation of crucial target substrates and S phase progression have been documented and characterized in work described in the previous Annual Report. Future work includes extension of *in vitro* studies to identify functional domains of c-Myc required for its estrogen-mediating effects.

ANNUAL SUMMARY

The aims of this project as outlined in the original application were to address several hypotheses arising from previous work highlighting the importance of c-Myc in estrogen stimulation of proliferation. These were that:

- Induction of c-Myc may precipitate a cascade of molecular events which mimic the effects of estrogen treatment i.e. c-Myc expression is *sufficient* to mimic the effects of estrogen.
- Activation of cyclin E-Cdk2 is a key element in both Myc- and estrogen-induced cell cycle progression and may occur by the same mechanism.
- c-Myc induction may be *necessary* for estrogen effects on molecular events leading to cell cycle progression, including cyclin E-Cdk2 activation.
- Given the essential requirement for estrogen in mammary gland development, c-Myc function may be also required for development of the mammary ductal tree

Testing these hypotheses has been undertaken as follows:

1. *Sufficiency of c-Myc expression for overcoming antiestrogen-induced arrest* (Task 1 in Statement of Work)

Clonal cell lines expressing c-Myc under the control of a zinc-inducible promoter were used to demonstrate that c-Myc can mimic estrogen effects on cyclin E-Cdk2 activation and cell cycle re-entry, and initiates a pathway which is initially distinct from that activated by cyclin D1 but converges with cyclin D1-dependent events at the activation of cyclin E-Cdk2. These experiments were described in detail in the previous Annual Report and have been published (9).

Since beginning this study, further details about the transcriptional regulatory activities of c-Myc have been elucidated. c-Myc is a bHLH/LZ (basic helix-loop-helix/leucine zipper) transcriptional regulator which forms heterodimeric complexes with another bHLH/LZ protein, Max. Myc can both activate and repress transcription and these functions require the amino terminus of c-Myc which contains two highly conserved Myc box regions. MbI (Myc box I, amino acids 45-63) is necessary for transcriptional activation; and MbII (amino acids 129-141) for transcriptional repression, c-Myc-induced cell proliferation and transformation (2-5). This raises the question of whether c-Myc-regulated transcriptional activation, repression or both are involved in estrogen stimulation of cell proliferation. Since MbII is necessary for c-Myc-induced cell proliferation in other systems we predict that c-Myc repressed genes are likely to be involved in both estrogen- and Myc-induced cyclin E-Cdk2 activation and G_1-S phase progression. However, it is not clear whether c-Myc activated genes are also likely to be involved.

In an extension of our original research plan we plan to test this by examining the ability of various c-Myc mutants to promote cell cycle progression in antiestrogen-arrested MCF-7 cells, using an experimental design similar to that used to demonstrate that full-length c-Myc can mimic the effects of estrogen. To this end we obtained the following c-Myc mutants from other investigators: cMycΔMbII, which has a deleted MbII domain (1); and MycS, a naturally occuring translational form of c-Myc, which lacks 100 amino-terminal amino acids, including the MbI domain and cannot activate transcription but can stimulate proliferation (11, 12). We also have a construct lacking both Myc boxes, c-*zip* (6). This has dominant-negative activity and thus has been used in complementary experiments aimed at determining whether c-Myc function is necessary for estrogen-induced mitogenesis (see below). In the present experiments it serves as a negative control.

The c-Myc mutants were all obtained in constitutive mammalian expression vectors and thus the initial experimental approach chosen was to transiently transfect these constructs into MCF-7 cells together with a marker plasmid (eg. green fluorescent protein (GFP)), and analyse rescue of the productively transfected cells into S phase by dual parameter flow cytometry after counterstaining for DNA content. Experiments within the laboratory had shown that transfection efficiencies of the order of 25% could be achieved using Fugene6, a marked improvement over other transfection techniques. Preliminary experiments using several marker plasmids showed that GFP was not a suitable marker since it was not possible to retain the GFP fluorescence while achieving acceptable DNA staining, probably due to leakage of cytoplasmic GFP after fixation. However, CD20, which is commonly used in such experiments since it is a surface marker normally expressed only on B-cells, proved more suitable. Surface immunofluorescent detection of transfected CD20 expression (using FITC-labelled antibodies) together with DNA staining using propidium iodide yielded good quality DNA histograms whilst retaining CD20 positivity. Using this approach, however, we were unable to demonstrate rescue of antiestrogen arrest by full-length c-Myc, in contrast with results obtained in cell lines permanently transfected with inducible c-Myc constructs. These experiments were hampered by technical difficulties in reliably obtaining good transfection efficiencies in arrested cells, but nevertheless expression of transfected c-Myc was readily apparent by Western blotting. Since high levels of c-Myc can induce apoptosis and in the constructs used c-Myc was under the control of a strong viral promoter it appeared likely that cells transiently overexpressing c-Myc were undergoing apoptosis rather than proliferation. Some evidence for increased apoptosis

was obtained, supporting this interpretation, and this experimental approach was therefore abandoned.

Given the previous success within the laboratory with zinc-inducible constructs (7, 9), it was decided to use this approach for expressing the c-Myc mutants. The various mutant c-Myc cDNAs have now been cloned into pΔMT, the vector used in our previous studies, downstream of the metallothionein promoter. Experiments to date have shown protein expression from these vectors and it is planned to begin selection of permanently-transfected clonal cell lines expressing these constructs in the near future. These will be used to determine whether Myc-mediated transactivation, repression or both are sufficient to mimic estrogen effects on parameters including cyclin E-Cdk2 activation and entry into S phase. If time permits, these studies may be extended to identify Myc-responsive genes important for estrogen effects, for example by the use of DNA microarrays.

2. *Necessity of c-Myc expression for estrogen-induced cell cycle progression* (Task 2 in Statement of Work)

These experiments are complementary to those described above and utilise overlapping approaches. At the time of the last Annual Report we were experimenting with the use of 'TAT-tagged' proteins as a means of introducing dominant-negative proteins into estrogen-stimulated MCF-7 cells. This method utilises the ability of a 9 amino acid tag derived from HIV TAT to facilitate passage of heterologous proteins across the cell membrane (8). TAT fusion proteins are expressed in bacteria, purified and denatured then added to the cells of interest, where they cross the cell membrane and refold into an active conformation. Despite input from the lab which originally developed this methodology we were, however, unable to demonstrate biological effects of either Myc-TAT proteins or the p27-TAT construct used by Nagahara et al (8) in MCF-7 cells.

Other colleagues within the laboratory have been investigating the use of antisense oligonucleotides to reduce c-Myc function. These experiments are ongoing and, if specific inhibition of c-Myc function can be achieved, the technique will be utilised to examine the necessity for c-Myc function in estrogen-induced cell cycle progression. Thus, cells will be treated with antisense oligos during or immediately prior to estrogen 'rescue' of antiestrogen-arrested MCF-7 cells.

Experiments in progress are aimed towards using inducible expression of proteins which dominantly inhibit c-Myc function, as described above. cDNAs encoding two proteins with previously-demonstrated dominant negative activity have been obtained. These are: c-*zip*, a c-Myc mutant lacking the transactivation domain; and rep-max, a synthetic chimera of the mSin interaction domain of Mxi and the bHLH-LZ region of Max (10). Dimerisation with Max is obligatory for the biochemical and biological activities of c-Myc and both these dominant-negative proteins dimerise with Max and therefore compete with endogenous c-Myc but do not regulate c-Myc-responsive genes. These are currently being cloned into the zinc-inducible vector pΔMT for use in experiments paralleling those described in part 1 above. In this case, however, rather than testing the ability of the constructs to 'rescue' antiestrogen-arrested cells, the dominant negative proteins will be induced during estrogen-rescue.

3. *Role of c-Myc in normal mammary gland development* (Task 3 in Statement of Work)

Here it was planned to use the method of 'tissue recombination', whereby primary cultures of normal murine mammary epithelial cells are infected *in vitro* using a retrovirus expressing the gene of interest. These cells are then reintroduced into the 'cleared' mammary fat pad (i.e. lacking endogenous mammary epithelium) of another mouse where they develop into a morphologically normal mammary ductal tree. Specifically, it was planned to express c-Myc dominant-negative proteins and determine whether this interfered with the ability of cells to form a mammary ductal structure. This part of the project is dependent on successful completion of the experiments described in Part 2 above and thus has not yet been initiated. Further consideration of the experimental approach in consultation with colleagues familiar with the technique has identified a number of potential technical problems which it is felt will preclude successful completion of these experiments. The major problem is the likelihood that, assuming c-Myc function is indeed necessary for mammary development, untransfected mammary cells will overgrow the dominant-negative expressing cells to such a degree during development of the mammary tree that the dominant-negative expressing cells will be impossible to identify. It may be feasible to select the transfected cells before reimplantation but this is likely to impair the ability of the transplanted cells to regrow. Other alternatives, for example transgenic expression of the dominant negative constructs have been considered but are unlikely to be completed during the granting period. Therefore, it is planned to focus on the *in vitro* approaches for the remaining year of funding.

KEY RESEARCH ACCOMPLISHMENTS

- Development and validation of technique for determining cell cycle phase distribution of transiently transfected MCF-7 cells
- Evaluation of TAT-mediated transduction as a means of introducing c-Myc mutants into MCF-7 cells
- Demonstration that constitutive expression of c-Myc is not an appropriate experimental strategy for the current experiments
- Construction of vectors for inducible expression of various c-Myc mutants

REPORTABLE OUTCOMES

Manuscripts

1. Prall OWJ, Rogan EM, Musgrove EA, Watts CKW, Sutherland RL. (2000) Estrogen regulation of cell cycle progression. **Hormonal Carcinogenesis III** pp. 220-227 Li JJ, Daling JR, Li SA, eds. Springer-Verlag, New York

2. Watts CKW, Prall OWJ, Carroll JS, Wilcken NRC, Rogan EM, Musgrove EA, Sutherland RL. Antiestrogens and the cell cycle **Antioestrogens and antiandrogens** In press Jordan VC, Furr BJ, eds. Humana Press.

Invited Presentations

1. Sutherland RL. Control of cell cycle progression by estrogen and its antagonists. Sixth International Congress on Hormones and Cancer, Jerusalem, Israel, Sept 5-9, 1999.
2. Musgrove EA. G_1 cell cycle regulators in breast cancer. Third PeterMac Symposium: Initiation and progression of cancer, Melbourne, Vic, Australia, Nov 7-10, 1999.
3. Sutherland RL. Antiestrogen-estrogen control of cell cycle progression. 14th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology, June 24-27, 2000.

CONCLUSIONS

During the course of this grant we have demonstrated that c-Myc is sufficient to initiate re-entry of antiestrogen-arrested cells into the cell cycle, thus mimicking the effects of estrogen, and that this occurs via a pathway that is initially distinct from cyclin D1-activated events. However, both pathways converge on activation of cyclin E-Cdk2. Ongoing experiments are aimed at identifying which functions of c-Myc (i.e. activation, repression or both) are required to mimic the effects of estrogen, and determining whether activation of c-Myc is *necessary* as well as *sufficient*. A number of technical problems have been encountered to date but we are confident that these have now been resolved and the key results will become available during the final year of the grant.

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